# Changes in ATP Content in Cerebellar Granule Cells during Hyperstimulation of Glutamate Receptors: Possible Role of NO and Nitrite Ions

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 143, No. 4, pp. 419-422, April, 2007
Original article submitted September 22, 2006

In primary 7-8-day culture of cerebellar granule cells, glutamate exposure (100 μM, 10-240 min) induced a 60-30% drop in ATP level; during the postglutamate period ATP level completely recovered after 24 h. Inhibition of NO-synthase with L-NAME during glutamate application resulted in less pronounced decrease in ATP level immediately after its application and had no effect on ATP recovery after 24 h. It was found that hyperstimulation of glutamate receptors elevates concentration of NO products (nitrites and nitrates), while $NO_2^-$ ions can increase ATP content.

**Key Words:** *neurons, glutamate; ATP; NO; nitrites*

The decrease in ATP content in neurons during brain hypoxia and hyperstimulation of glutamate receptors can disturb intracellular and intercellular signaling in cerebral neurons, in particular, ionic homeostasis, activity of glycolytic and oxidative phosphorylation enzymes, mitochondrial $Ca^{2+}$ uptake, and protein synthesis [6-8,10,11,13]. Previous studies showed that ATP content in cultured cerebellar granule cells (CGC) decreased immediately after application of glutamate (Glu) [1,2,8,3]. However, the dynamics of ATP content in postglutamate period is poorly studied.

Elevated content of NO is a potential factors promoting Glu-induced decrease in ATP concentration [10,11]. Examination of the effects of exogenous NO donors on energy metabolism in neurons showed that NO in high concentrations inhibits both oxidative phosphorylation and glycolysis [11]. At the same time, experiments on isolated rat mitochondria showed that $NO_2^-$ ions turn into NO under hypoxic condition and can accept electrons from cytochrome oxidase of the respiratory chain [4]. Here we examined the dynamics of ATP content in cultured CGC during application of GLU and in the postglutamate period and the role of NO and nitrites in this process.

## MATERIALS AND METHODS

Experiments were carried out on 7-8-day culture of rat CGC. The procedure of isolation of cell suspension for neuron culturing was described elsewhere [3,5,6]. The cells in culture were washed with a control solution containing (in mM): 130 NaCl, 5.6 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 20 HEPES, 5.0 glucose, pH 7.4. Application of Glu (100 μM) and inhibition of NO-synthase with 100 μM L-NAME ($N^{\omega}$-nitro-L-arginine methyl ester) was performed a $Mg^{2+}$-free solution containing 10 μM glycine at 20-25°C. The cultures were divided into two groups. In the first group, ATP content was determined immediately after the end of Glu application. Group 2 cultures were washed with control solution, incubated in neurobasal medium (NBM) with B-27 supplement

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0007-4888/07/1434-0442 

(Gibco) in a $CO_2$-incubator, and after 4 and 24 h washed from NBM with control solution. ATP was extracted with 2% trichloroacetic acid containing 2 mM EDTA. The extracts were neutralized with 3 M KOH/1.5 M Tris, centrifuged, and an aliquot of the supernatant was sampled for measurement of ATP concentration by luminescent method with luciferin-luciferase (Calbiochem) in 0.1 M Tris-acetate buffer at pH 7.75 [13]. The content of ATP was standardized by protein content in the sample. Protein concentration was determined with a special reagent (Bio Rad) after addition of 0.1 N NaOH to cell. The results were presented as percent of control (6.8±0.5 nmol/mg protein, 100%).

The content of NO products (nitrites and nitrates) in cultured CGC was measured after sedimentation of proteins using a Calbiochem kit, which converted nitrites into nitrates under the action of nitrate reductase followed by measurement of nitrite content with Griss reagent (according to manufacturer's instruction).

The concentration of cyclic guanosine monophosphate (cGMP) was determined by enzyme immunoassay in 0.4 N $HClO_4$ extracts neutralized with KOH [3]. The data were obtained on several cultures ($n$=3-4) in each experiment (no less than 3 for any index).

## RESULTS

We studied changes in ATP content in CGC depending on the duration of Glu application (Fig. 1). Immediately after 10-min Glu exposure, ATP content decreased to 62±10% of the control. Longer exposure (Fig. 3, *a*) induced more pronounced decrease in ATP level: to 47±5% (30 min), 40±4% (2 h), and 36±2% (4 h). Since the decrease in ATP concentrations was similar after 30-min, 2-h, and 4-h exposure with Glu, most measurements were performed after a 30-min exposure. During 24-h postglutamate period, ATP content returned to virtually control level (Fig. 1, *b*). Similar data on the recovery of energy level in CGC were obtained previously [8]. By contrast, in cortical neurons ATP level did not recover during the postglutamate period [7].

Glu-induced elevation of $[Ca^{2+}]_i$ activates NO-synthase and increases the content of endogenous NO [5,10]. In its turn, NO activates soluble guanylate cyclase and elevates cGMP concentration [14]; under these condition the NO-cGMP pathway plays an important role in protection of cerebellar neurons from neurotoxins [15]. We showed that NO content (determined from concentration of end products nitrates and nitrites) considerably increased immediately after Glu application (Fig. 2, *a*). The concentration of cGMP increased as early as during the first minute of Glu application and remained high after 15-min exposure. Similar increase in cGMP level was observed after application of $NaNO_2$ (Fig. 2, *b*). Since NO is the major activator of soluble guanylate cyclase involved in cGMP synthesis [14], our findings suggest that in cerebellar cultured neurons nitrites can be converted into NO.

Inhibition of NO-synthase with 100 μM L-NAME during 30-min application of Glu increased ATP content by 20% compared to the effect of Glu alone. We previously demonstrated that L-NAME prevented neuronal death induced by hyperstimulation of glutamate receptors [5]. It can be hypothesized that the increase of ATP concentration observed during inhibition of NO synthesis can protect neurons from the toxic effects of Glu. Inhibition of NO

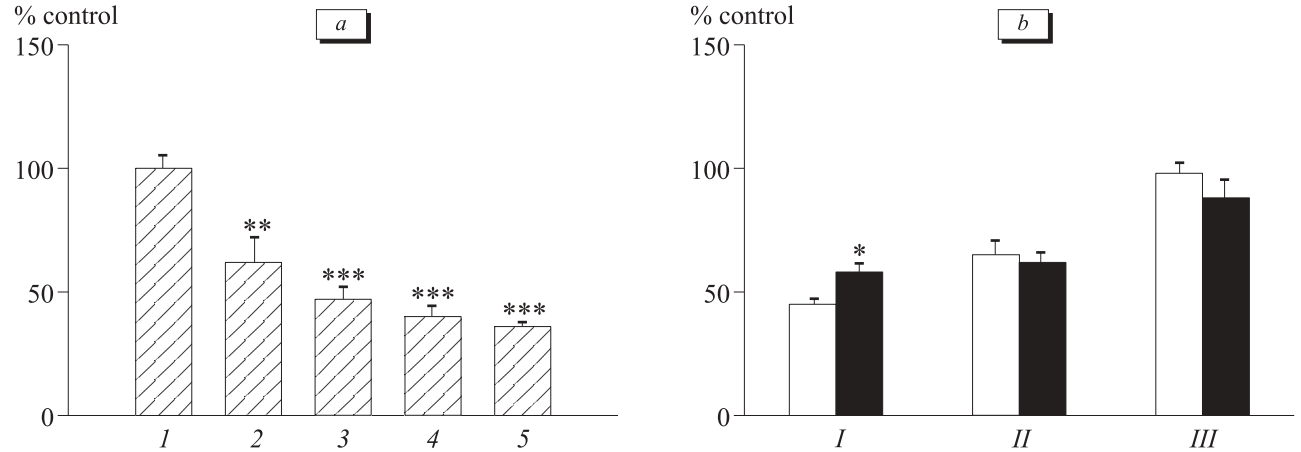


**Fig. 1**. Effect of hyperstimulation of glutamate receptors on ATP level in CGC culture. *a*: ATP content in the control (*1*) and after after 10-min (*2*), 30-min (*3*), 2-h (*4*), and 4-h (*5*) exposure with glutamate (100 μM); *b*: ATP content in the presence of NO synthase inhibitor after 30 min (*I*) and after exposure with 100 μM Glu (open bars) and 100 μM Glu with 100 μM L-NAME (dark bars) after washout with control solution followed by 4-h (*II*) and 24-h (*III*) incubation. *$p$<0.05, **$p$<0.01, ***$p$<0.001 compared to the control.

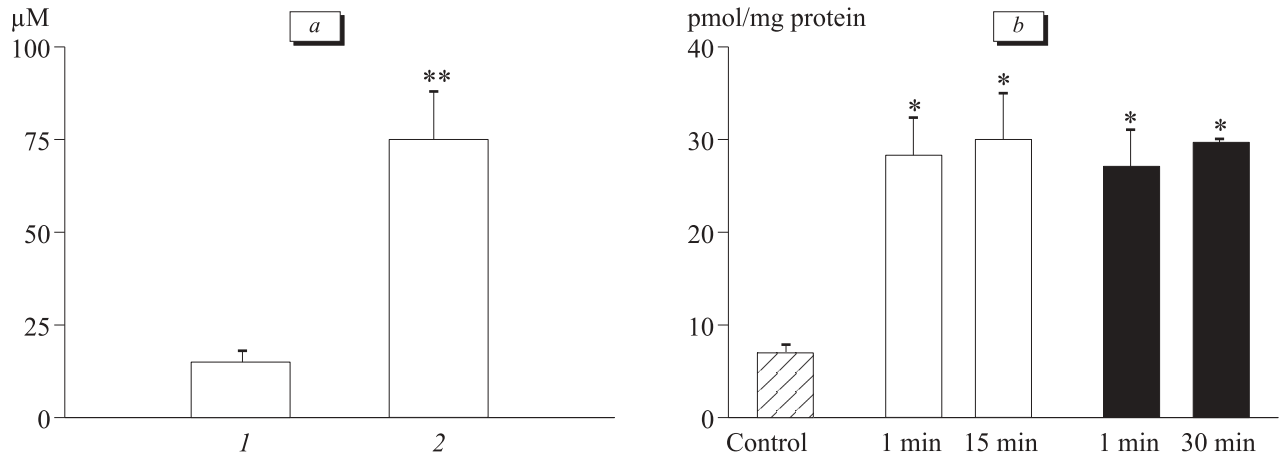


**Fig. 2**. Effect of Glu on production of NO metabolites and cGMP in CGC culture. *a*: effect of Glu on the content of NO products in the control (*1*) and after 30-min exposure with 100 μM Glu (*2*); *b*: cGMP level after application of 100 μM Glu (open bars) and 100 μM $NaNO_2$ (dark bars). **p*<0.01, ***p*<0.001 compared to the control.

synthesis during the postglutamate period had no effect on the dynamics of ATP concentration. Thus, our findings suggest that increased NO content affects the decrease in ATP concentration only during Glu application, whereas during the postglutamate period the difference in the decrease in ATP concentration did not depend on inhibition of NO synthesis during Glu application.

NO is a short-living molecule, which is rapidly converted into nitrites and nitrates. Since nitrite ions can accept electrons from the respiratory chain [4], it is important to find out whether these ions affect ATP level in cerebellar neurons.

Addition of 100 μM $NaNO_2$ after 15-min Glu application followed by their 15-min combined exposure significantly increased the content of ATP compared to the effect of Glu alone (Fig. 3, *a*). In concentrations below 100 μM, $NaNO_2$ and NO-regenerating compound nitrosocysteine (SNOC) significantly increased ATP level in the delayed period after exposure (after 4 h, Fig. 3, *b*). This increase in ATP content can be explained by acceptance of electrons from cytochrome oxidase by $NO_2^-$ ions after addition of $NaNO_2$ or formation of these ions due to dissociation of SNOC (100 μM) and stimulation of ATP synthesis in mitochondria. However, in higher concentration (1000 μM) SNOC significantly decreased ATP level compared to control samples. Increasing $NaNO_2$ concentration to 1000 μM also decreased ATP level. This significant drop of ATP content under the action of SNOC in high concentrations can result from inhibition not only in IV, but also in I and II complexes of the respiratory chain by NO [10]. Under these conditions, the ability of $NO_2^-$ to accept electrons from the respiratory chain cannot affect the increase in ATP level.

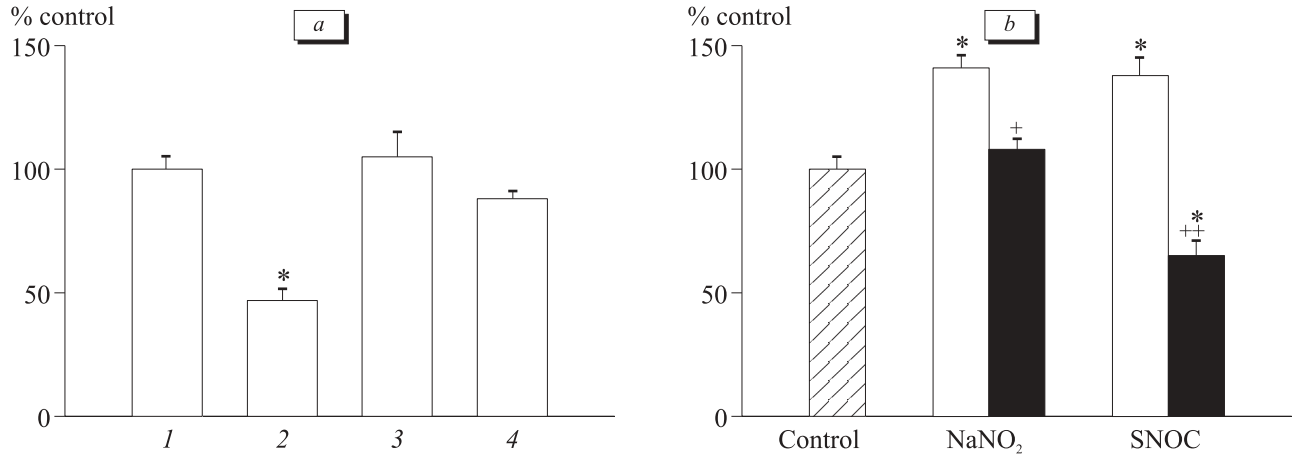


**Fig. 3**. Effect of $NaNO_2$ and SNOC in different concentrations on ATP content in CGC culture. *a*: ATP content in control (*1*) after 30-min application of 100 μM Glu (*2*) or $NaNO_2$ (*3*), and after 15-min application of 100 μM with combined application of both Glu and $NaNO_2$ (*4*); *b*: ATP level 4 h after 30-min exposure to $NaNO_2$ and SNOC in concentrations of 100 μM (open bars) or 1000 μM (dark bars). **p*<0.001 compared to the control; $^+p$<0.05, $^{++}p$<0.001 compared to the effect of the corresponding agent in concentration of 100 μM.

This explains opposite effects of 100 and 1000 μM SNOC on ATP synthesis in cerebellar neurons.

It is now accepted that activity of cytochrome oxidase complex (Green IV complex) is an endogenous marker of neuronal activity. Short-term inhibition of complex IV in respiratory chain by NO protects mitochondria by maintaining their membrane potential $\Delta\Psi_m$ thereby protecting cells from apoptosis [9]. Maximum activity of cytochrome oxidase in neurons is detected in close proximity to NO-synthase and NMDA receptors [10].

In mitochondria of plants and microorganisms, $NO_2^-$ ions can play the role of electron acceptors from the respiratory chain. It is also known that these ions can accept electrons from mitochondrial cytochrome oxidase [4]. Under conditions of hypoxia and hyperstimulation of glutamate receptors, accumulation of nitrites as NO end products can promote alternative ATP synthesis and contribute to ATP recovery in cerebellar neurons.

Thus, we demonstrated that hyperstimulation of glutamate receptors induces a short-term drop in ATP level, which recovered within 24 h after exposure. We also revealed a dual role of NO and $NO_2^-$ ions in energy metabolism of cerebellar neurons. The decrease in ATP content due to endogenously produced NO takes place only during Glu application and can be explained by reversible binding of NO with heme iron in cytochrome oxidase. Then, NO molecules are converted into nitrites and probably play a role of alternative (to oxygen) acceptors of electrons and restore ATP level in cerebellar neurons. At the same time, large amounts of exogenous NO can inhibit various elements of the respiratory chain and decrease ATP level.

This work was supported by the Russian Foundation for Basic Research (grant No. 05-04-49481) and by Leading Scientific Schools Grant of President of Russia (grant No. NSh-9124.2006.4).

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